(12) UK Patent Application (19) GB (11) 2 288 232 (13) A

(43) Date of A Publication 11.10.1995

(21) Application No 9406737.8

(22) Date of Filing 06.04.1994

(51) INT CL[6]
G01N 21/76, C12Q 1/66

(52) UK CL (Edition N)
G1B BAB BAH B122 B626 B639 B719

(56) Documents Cited
EP 0233403 A1

(58) Field of Search
UK CL (Edition M) G1B BAB BAH BAX BBS
INT CL[5] C12Q 1/66, G01N 21/76
ONLINE DATABASE: WPI, BIOTECH (DIOLOG)

(71) Applicant(s)
BRF International

(Incorporated in the United Kingdom)

Lyttel Hall, Coopers Hill Road, Nutfield, REDHILL, Surrey, RH1 4HY, United Kingdom

(72) Inventor(s)
William J Simpson
Julian M Pye

(74) Agent and/or Address for Service
Urquhart-Dykes & Lord
91 Wimpole Street, LONDON, W1M 8AH, United Kingdom

(54) **Photosensitive derivatives of ATP as assay control standards**

(57) There is disclosed the use of a photosensitive derivative of adenosine triphosphate (ATP) for use in the bioluminescent assay of ATP. The derivative may be caged-ATP e.g. nitrophenol ester of ATP which contains a photohydrolysable ester bond. The sample which is being assayed for ATP is mixed with a reagent containing luciferin-luciferase and a known quantity of caged-ATP and the light emitted is measured. The mixture is then exposed to a flash of intense light resulting in the release of ATP in a photolytic reaction. This ATP reacts with the firefly luciferase to emit light, thus allowing the amount of the sample ATP to be deduced. The method overcomes problems with contaminated or unstable standard solutions of ATP.

GB 2 288 232 A

The claims were filed later than the filing date within the period prescribed by Rule 25(1) of the Patents Rules 1990.

2288232

METHOD FOR CALIBRATING ATP ASSAYS

The present invention relates to a method for calibrating an assay for adenosine 5'-triphosphate (ATP).

Detection of contaminant microorganisms for the purposes of product or process control, or for food safety reasons, is of paramount importance to the food and beverage industries. Microbial biomass estimation is also important in a number of other applications, including control of waste processing, monitoring of sterilization processes and monitoring of air quality. In many cases, it is advantageous to be able to estimate the degree of microbial or process contamination within the shortest possible period of time. An ATP-bioluminescence technique, can provide a meaningful result in less than a minute and accordingly fulfils many of the criteria on which such rapid methods are judged.

Adenosine 5'-triphosphate (ATP) is found in live cells, but not in dead cells. In the presence of a purified enzyme (luciferase) from the American firefly, *Photinus pyralis*, a substrate, D-luciferin, and sufficient magnesium ions and dissolved oxygen, the following reaction takes place:

$$\text{Mg–ATP} + O_2 + \text{D–luciferin} \xrightarrow{\text{firefly luciferase}} \text{decarboxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

(ATP = adenosine 5'-triphosphate; Mg-magnesium ions; O_2 = oxygen; AMP = adenosine 5'-monophosphate; PP_i = inorganic phosphate; CO_2 = carbon dioxide).

Under appropriate conditions, the amount of light produced

by the reaction is directly proportional to the ATP concentration and can be detected using a sensitive light detector. This is the basis of the ATP-bioluminescence assay.

In order to maximize the accuracy of such assays, standardization of the assay is important.

Assays for ATP using the firefly luciferase reaction can be calibrated in two ways (Jago, P.H., Stanfield, G. Simpson, W.J. & Hammond, J.R.M. 1989. In ATP Luminescence: Rapid Methods in Microbiology, Society of Applied Bacteriology Technical Series, Vol. 26, Stanley P.E. et al. (eds) pp 53-61). According to an external standardization technique, the light output from reaction of the firefly luciferase reagent with the sample can be compared to that obtained from reaction of the firefly luciferase reagent with known amounts of ATP using a standard curve. While this technique is convenient and requires the minimum of reagent manipulation, it is also subject to error. This is because the light output from firefly luciferase reactions is directly related to the rate of reaction. While the ATP concentration does, indeed, make a major contribution to controlling the reaction rate, it is not the sole determinant. Inhibitors present in the sample (eg. metal ions, hydrogen ions) can reduce reaction rate, and stimulators such as detergents can increase reaction rate (Simpson, W.J. and Hammond, J.R.M. 1991. *Journ. Chemilumin. & Biolumin.*, 6, 97-106). In addition, the inherent activity of the firefly luciferase reagent itself may vary due to inconsistencies in production or handling. Even though the reaction rate may be unaffected, there may be substances present in the reaction mixture which absorb the light produced to a significant extent, thus introducing error. Some substances, notably Zn^{2+}, in addition to reducing the catalytic activity of firefly luciferase also change the wavelength of the light produced in the reaction. This can cause a reduced amount of light to be detected from

the reaction in some types of luminometers (Denburg, J.L. & McElroy, W.D. 1978. *Arch. Biochem. Biophys.* **141**, 668-675).

All of these sources of error can be eliminated if the standard curve approach is abandoned in favour of the internal standardization technique. In its simplest form, this consists of adding a known amount of ATP, contained in a small volume of liquid, to an initiated firefly luciferase reaction. The light output from sample and luciferase alone, and from sample and luciferase and ATP are compared, and the ATP content of the sample then calculated. This method frees the operator of analysis errors associated with variable sample composition. The cornerstone of this technique, however, is the use of a stable ATP standard solution. A great deal of controversy exists with regard to the stability of such ATP standard solutions. Some workers claim that such dilute solutions of ATP are unstable and must be kept away from light and/or stored on ice. Also problems can be experienced due to poor handling of the solution. By far the largest threat to the stability of ATP standard solutions is the presence of contaminant microorganisms in the solution. ATP is rapidly utilized by many microorganisms (Karl, D.M. 1980. *Microbiol. Rev.* **44**, 739-796), and therefore their exclusion from ATP standard solutions is essential. Aseptic technique is mandatory. Although pre-weighed vials of ATP can be purchased from some specialist luminescent reagent manufacturers, their use is not widespread, on account of these perceived limitations. These problems become all the more important when ATP-bioluminescence assays are employed in safety-critical areas such as monitoring of food hygiene and air quality.

It is an object of the present invention to ameliorate the above problems in standardizing ATP assays.

According to the present invention there is provided a method for calibrating an ATP bioluminescence assay

comprising the steps:

1) Incorporating a photosensitive derivative of ATP into a firefly luciferase luciferin reagent at a pre-determined concentration;
2) Mixing a sample to be assayed and the reagent;
3) Measuring the light emitted by the luminescent reaction;
4) Exposing the sample/reagent mixture to a flash of visible light of high intensity;
5) Measuring the light emitted from the luminescent reaction;
6) Calculating the amount of ATP present in the mixture.

According to the present invention there is also provided a kit for calibrating an assay of ATP, the kit comprising:

a) An assay buffer;
b) A photosensitive derivative of ATP; and
c) A firefly luciferase - luciferin reagent.

The present method, a photostandardization technique, provides several advantages over previously available standardization methods in respect of (i) precision and accuracy of results; (ii) verification of results; and (iii) user friendliness. Enhanced precision results from the elimination of pipetting errors associated with ATP standard addition, and from compensation for any changes in optical or catalytic quenching associated with assay dilution. The ability to apply a simple, valid standardization protocol is likely to give benefits in the form of facilitated result verification, a point of particular significance with respect to Food and Standards legislation and certain safety-critical applications. Improved user friendliness results from the facts that no separate ATP standard solution is necessary to perform such assays, and the assay reagents can be factory calibrated.

A range of synthetic compounds of biological interest has been developed for use in physiological studies, which

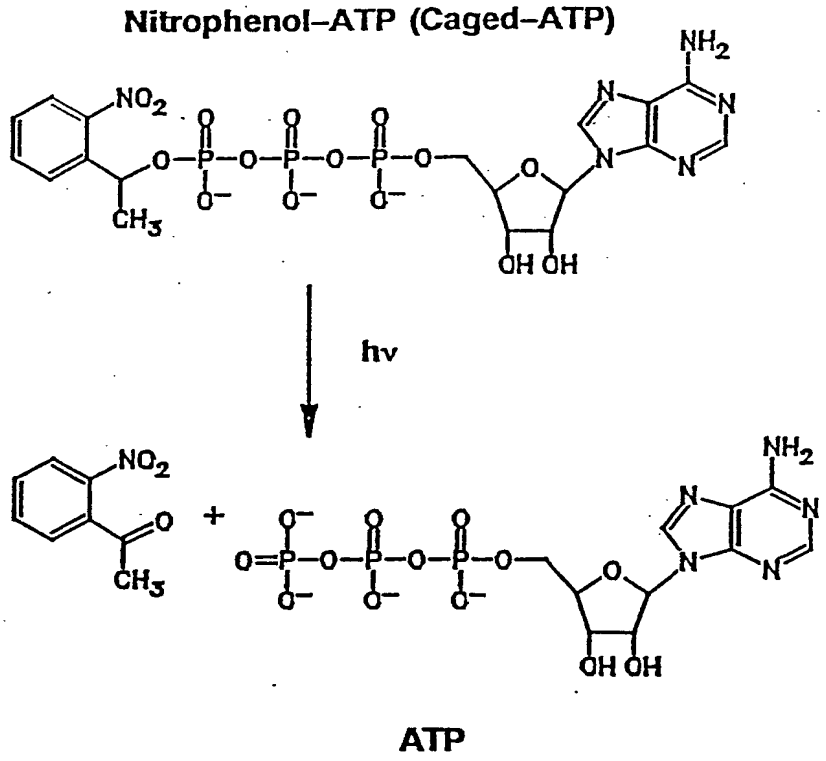
contain a photo-sensitive chemical bond. Thus, when one of these compounds is exposed to a brief flash of intense light, a reaction product is released. The photo-sensitive compound is referred to as a "caged" compound, from which a molecule or ion of interest can be liberated. Hence, ATP is released from caged-ATP, Ca^{2+} is released from caged-Ca^{2+}, and so on. Various derivatives of ATP have been synthesized which release ATP in this way. One example of these ATP derivatives is a nitrophenol ester of ATP, which contains a photohydrolysable ester bond (Figure 1). The development of these substances has allowed studies on the effect of rapid ATP release in physiological specimens to be determined. In such experiments, the compound is introduced into the cells by micro-injection then the ATP released from the cage, using a high intensity photoflash lamp (Gurney, A.M. & Lester, H.A. 1987, *Physiological Reviews*, 67, 583-617.,).

Caged-ATP has been used in a number of research applications relating to a variety of areas, including the study of muscle biochemistry (Goldman, Y.E., Hibbard, M.G. McCray, J.A. & Trentham, D.R. 1982, Nature, 300, 701-705). It has not been used previously for the purpose of calibrating biochemical or microbiological assays.

The yield of ATP from caged-ATP has been shown to be independent of pH over the pH range 6-9; furthermore, caged-ATP has been shown to be convenient to work with in that it is soluble in water at neutral pH and is not significantly photolyzed during several minutes exposure to subdued daylight (McCray, J.A. Herbette, L. Kihara, T. & Trentham, D.R. 1980, Proc. Natl. Acad. Sci., USA, 77, 7237-7241).

Such a photosensitive ATP derivative is suitable for use in the present invention.

Figure 1 Release of ATP from caged-ATP



The use of such a photosensitive derivative of ATP, together with a high intensity photolamp provide a convenient and non-invasive means of standardizing ATP-bioluminescence assays. The derivative of ATP can be one of several compounds, referred to generically as "caged-ATP". This invention was developed using one of these compounds, a nitrophenol ester of ATP. Other members of this group of compounds could be substituted for the nitrophenol-ATP ester in this process.

Commercially-available supplies of caged-ATP are contaminated with ATP giving rise to significant luminescence when they are first introduced into a luciferase-luciferin reagent. This can be reduced to a technically insignificant level in two ways. (i) The luciferin-luciferase reagent containing the caged-ATP can be incubated for 8-20 hours or more at 4°C. During this time, firefly luciferase catalyses hydrolysis of ATP but does not affect the level of caged-ATP. (ii) The concentration of contaminant ATP present in the reagent can be reduced by treatment with an ATP-destroying enzyme such as apyrase, either in a soluble form or in an immobilized form.

The caged-ATP compound is incorporated into a firefly luciferase reagent at a pre-determined concentration. Firefly luciferase does not display any catalytic activity toward caged-ATP (see Example 1), and therefore in the absence of added ATP, no light is produced from such a luciferase preparation. The reagent is used to perform highly sensitive assays of ATP in the following way. Sample and reagent are mixed in a disposable luminometer cuvette. The sample may consist of an aqueous fluid of any origin or may be a specially prepared "extract" produced as a result of treatment with any of a variety of agents, designed to release ATP from living organisms. The light emitted from the luminescent reaction is measured using a luminometer. A known amount of ATP is

then released into the reaction mixture by exposing the cuvette together with its contents to a flash of visible light of high intensity, delivered from a photoflash lamp. The cuvette is then placed back into the luminometer and the light emission from the luciferase reaction measured once again. Since the light flash causes the release of a known amount of ATP from the caged-ATP present, the amount of light attributable to the presence of this known amount of ATP can be calculated by difference. The amount of ATP originally present in the reaction mixture can then be calculated.

The following non-limiting Examples are intended to describe the nature of the invention more clearly:-

Example 1 Use of caged-ATP for standardization of ATP assays

Caged-ATP (Calbiochem, USA, prod. no. 119127,5 mg) was dissolved in 0.5 ml sterile de-ionized water. The resulting stock solution was stored frozen at -20°C until required. Before use, it was diluted 1 in 1000 with sterile de-ionized water. To 3 ml of a commercially-available luciferase-luciferin reagent (Biotrace XT reagent, Biotrace Ltd., Bridgend, UK) was added 200 µl of sterile de-ionized water to form a control reagent or 200 µl of the caged-ATP stock solution to form a caged-ATP reagent. The reagents were then incubated for 1-6 hours at 20°C.

At various times, after preparation of the reagents, sterile de-ionized water (300 µl) was transferred to a clean disposable luminometer cuvette then 100 µl of control reagent or caged-ATP reagent added. The light emitted from the reaction was quantified using a Biotrace M3 Luminometer (Biotrace Ltd, Bridgend, UK), which integrated the light response for 10 seconds after a 2 second delay. A volume of ATP solution (10 µl, 0.1 µM) was then added to the reaction mixture and the light

output measured for a second time. The cuvette, together with its contents, was then exposed to an intense flash of light from a Hanimex 325A2 flash gun (Hanimex, UK) and the light output from the reaction measured for a third time.

The results in Table 1 show the light output values obtained. Several points are highlighted by these results: (i) caged-ATP did not compete with ATP for the active site on firefly luciferase as indicated by the similar response observed to 1 pmol added ATP recorded for both reagents (average, $\underline{n}$ = 5) of 1302 RLU from 1 pmol ATP in absence of caged-ATP and 1304 RLU from 1 pmol ATP in presence of 95.2 pmol caged-ATP); (ii) the high intensity light flash did not affect the rate of the firefly luciferase reaction as demonstrated by the fact that the response of the reagent to 1 pmol ATP was unchanged after flashing (1308 RLU before flashing, 1276 RLU after flashing: the difference can be solely attributed to the natural decay in luminescence associated with such reactions); (iii) exposure of the caged-ATP reagent to a high intensity flash of light produced an increase in ATP content in the assay, equivalent to 1.171 pmol ATP/assay. The assays could be calibrated on the basis of this response. Calibration of the assays in this way (with respect to the 1 pmol of free ATP added to the reaction) gave a value of 1.00 ± 0.08 pmol ATP/assay (mean ± S.D, $\underline{n}$ = 5) with the errors randomly distributed with respect to time. In the case of the control assays, standardized by the standard curve technique (using the data at a reagent age of 1 hour), a value of 0.93 ± 0.06 pmol ATP/assay (mean ±S.D, $\underline{n}$ = 5) was obtained, with the individual values showing a marked decrease with respect to time since reagent preparation (on account of a reduction in luciferase catalytic activity).

Once introduced into the luciferase reagent, caged-ATP might release ATP either by spontaneous hydrolysis, as a result of enzymic action, or by photolysis. The results shown in Example 1 indicate that release of ATP by the

former two mechanisms is negligible and that, on exposure to a high intensity flash of light produced by a flashgun, ATP is produced rapidly from the caged-ATP present.

Notes to Table 1

1. The high intensity photoflash (provided by a Hanimex 325A2 flash gun, Hanimex, UK) did not induce luminescence from empty cuvettes, nor from cuvettes containing test solutions such as water or caged-ATP, or luciferase-luciferin reagents in the absence of caged-ATP.

2. The "ATP photostandard" (caged-ATP) produced 1.171 pmol ATP/assay on exposure to a high intensity photoflash. This represents a 1.23% conversion rate of the caged-ATP present in the assay.

3. Similar results were obtained when the assays were performed in the presence of a detergent-based ATP extractant (swab diluent XT, Biotrace Ltd, Bridgend, UK), instead of water, as the sample matrix indicating that the photostandardization technique is compatible with the use of such reagents.

Example 2

In a second experiment, a similar set of assays was conducted. In these experiments, the control and caged-ATP luciferin-luciferase reagents had been incubated for 6 hours prior to use. The sample mix to which the reagent was added prior to addition of ATP and/or light flashing was sterile de-ionized water, or mixtures of water and 0.1 M sodium 3,3'-dimethylglutarate buffer (pH 4.00). These experimental conditions were designed to generate a range of pH values in the final assay. The results in Table 2 show that, in the presence of increasing amounts of buffer, the light output from reactions containing 1 pmol ATP was reduced. Thus, if the standard curve approach was

adopted and the results referred to those obtained using water as the sample matrix, substantial errors were evident. In the extreme, a value of 0.05 pmol ATP/assay was obtained, i.e. one-twentieth of the true value. Results based on the photostandardization technique were also subject to error but of a much smaller magnitude.

By increasing exposure of the reagents to light, for example by increasing the number of flashes, it is possible to release more ATP into the reaction (Table 3). Thus, it is possible to perform multi-point calibration of ATP assays by exposing the sample to light several times and recording the amount of light emitted from the luciferin-luciferase reaction in each case. Above a given light dose, the response curve saturates and due attention must be paid to this, in order to exploit the method to the fullest.

Table 1: Use of caged-ATP to standardize ATP assays over the course of a working day

Time since preparation of reagent (h)	LIGHT OUTPUT (RLU*)						CALCULATED ATP CONTENT (pmol/assay) OF STANDARD BASED ON	
	No added ATP		+ 1 pmol ATP		+ Light flash			
	control reagent	*caged-ATP reagent*	*control reagent*	*caged-ATP reagent*	*control reagent*	*caged-ATP reagent*	*(i) standard curve at T = 1h*	*(ii) photo-standardization*
1	7	69	1405	1495	1419	3129	1.00	1.02
2	7	20	1372	1377	1320	2875	0.98	1.06
4	6	57	1315	1367	1308	3014	0.94	0.93
5	6	80	1262	1258	1211	2786	0.90	0.90
6	5	24	1185	1271	1121	2616	0.84	1.09
						$\bar{x}$	0.93	1.00

* RLU = relative light unit

Table 2: Use of caged-ATP to standardize ATP assays at different pH values

Test Sample Mix	LIGHT OUTPUT (RLU*)						CALCULATED ATP CONTENT (pmol/assay) OF STANDARD BASED ON	
	No added ATP		+ 1 pmol ATP		+ Light flash			
	control reagent	*caged-ATP reagent*	*control reagent*	*caged-ATP reagent*	*control reagent*	*caged-ATP reagent*	*(i) standard curve at T = 1h*	*(ii) photo-standardization*
Sterile de-ionized water	6	24	1185	1271	1121	2616	1.00	1.09
Water/NaDMG† buffer (250: 50)	9	21	949	871	912	2427	0.80	0.64
Water/NaDMG† buffer (150:150)	10	13	378	430	392	1056	0.31	0.78
Water/NaDMG† buffer (0:300)	8	8	72	65	84	217	0.05	0.44

* RLU = relative light unit

† NaDMG = 0.1 M sodium 3,3'-dimethylglutarate buffer, pH 4.00

Table 3: Effect of light dose on release and subsequent reaction of ATP

Light dose to which sample was exposed (number of flashes)	Light emission from luciferin–luciferase reaction* (RLU)
0	215
3	15726
6	32772
9	40363
12	46260
15	52203
18	53663

* The reaction consisted of 40 µl caged–ATP solution (1.43×10^{-5} M) and 100 µl luciferin–luciferase solution

CLAIMS

1. A method for calibrating an ATP bioluminescence assay comprising the steps:-

i) incorporating a photosensitive derivative of ATP into a firefly luciferase-luciferin reagent at a predetermined concentration;

ii) mixing a sample to be assayed and the reagent;

iii) measuring the light emitted by the luminescent reaction;

iv) exposing the sample/reagent mixture to a flash of visible light of high intensity;

v) measuring the light emitted from the luminescent reaction; and

vi) calculating the amount of ATP present in the mixture.

2. A method as claimed in claim 1 wherein the photosensitive derivative of ATP is caged ATP.

3. A method as claimed in claim 1 or 2 wherein the luciferin-luciferase reagent containing the caged ATP is incubated for 8-20 hours or more at 4°c.

4. A method as claimed in claim 1 or 2 wherein the luciferin-luciferase reagent containing the caged ATP is treated with an ATP destroying enzyme.

5. A kit for calibrating an assay of ATP comprising;

a) an assay buffer;

b) a photosensitive derivative of ATP; and

c) a firefly luciferase-luciferin reagent.

Patents Act 1977 Examiner's report to the Comptroller under Section 17 (The Search report) 16	Application number GB 9406737.8
Relevant Technical Fields (i) UK Cl (Ed.M) G1B (BAB, BAH, BAX, BBS) (ii) Int Cl (Ed.5) G01N 21/76; C12Q 1/66	Search Examiner DR N CURTIS Date of completion of Search 14 NOVEMBER 1994
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE DATABASE: WPI, BIOTECH (DIALOG)	Documents considered relevant following a search in respect of Claims :- 1-5

Categories of documents

X:	Document indicating lack of novelty or of inventive step.	P:	Document published on or after the declared priority date but before the filing date of the present application.
Y:	Document indicating lack of inventive step if combined with one or more other documents of the same category.	E:	Patent document published on or after, but with priority date earlier than, the filing date of the present application.
A:	Document indicating technological background and/or state of the art.	&:	Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
A	EP 0233403 A1 (M.R.C.) see page 1, paragraph 1	1

Databases: The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).